Diagnosis of Pneumococcal Pneumonia by Enzyme-Linked Immunosorbent Assay of Antibodies to Pneumococcal Hemolysin (Pneumolysin)

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An enzyme-linked immunosorbent assay (ELISA) with a highly purified pneumolysin as the antigen was evaluated for serological diagnosis of pneumococcal pneumonia. One hundred four healthy controls were tested, and the specificity of the test was set to 95%. In samples from patients with bacteremic pneumococcal pneumonia, 82% (18 of 22) were positive, i.e., at least one serum sample had a titer above the upper normal limit or at least a twofold rise in antibody titers was noted. In nonbacteremic pneumococcal pneumonia, 45% (21 of 47) of samples were positive. All sera were negative for patients with pneumonia caused by *Haemophilus influenzae, Legionella pneumophila, Chlamydia psittaci,* and influenza A virus. However, in patients with a diagnosis of *Mycoplasma pneumoniae* infection, 8 of 25 (32%) samples were positive for antibodies to pneumolysin. All sera, including those from patients with mycoplasma infection, were negative to a protein control antigen by ELISA. Serum immunoglobulin G response to pneumolysin as measured by ELISA might thus be an aid in the laboratory diagnosis of pneumococcal pneumonia. This assay may also help to further elucidate the occurrence of dual infections with pneumococci.

Streptococcus pneumoniae is reported to be the most important causative agent of bacterial pneumonia. Laboratory verification of the diagnosis is, however, often difficult owing to the difficulty in obtaining adequate samples from patients and to the sometimes high number of healthy carriers of this organism (1, 4, 9, 14). The sensitivity and specificity of the methods for detecting pneumococcal antigens are far from 100% (9, 11). The only accepted method for definite diagnosis of pneumococcal pneumonia is blood culture, which is positive only in 20 to 25% of cases (1, 9, 14).

A simple, sensitive, and reliable serological assay would therefore be a valuable complement to the laboratory arsenal. Antibodies have been measured against the capsular polysaccharide antigens, but the number of prevalent antigen types and the occasionally low immunogenicity of the polysaccharides are evident drawbacks (2, 10, 16).

For staphylococcal and streptococcal infections, hemolysins have been used as antigens in serological assays, both as neutralization tests (15, 18) and as enzyme-linked immunosorbent assay (ELISA) (7). Most strains of *S. pneumoniae* produce an intracellular hemolysin, pneumolysin, which is similar to streptolysin 0 with respect to its inactivation by oxygen and cholesterol (6).

The aim of this study was to establish an ELISA for the detection of serum antibodies to purified pneumolysin and to evaluate the assay for the diagnosis of pneumococcal pneumonia in adult patients in relation to other diagnostic methods.

MATERIALS AND METHODS

Pneumolysin preparation. Pneumolysin was cultivated and purified as described in detail in an accompanying manuscript (13). Briefly, a clinical isolate of *S. pneumoniae* type

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14 was cultivated in a 10-liter fermentor, and the bacteria were disrupted by X-press (Biotec, Bromma, Sweden). The released pneumolysin was purified by ion-exchange chromatography, covalent affinity chromatography, and gel filtration. The purified preparation showed only one band in sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis and had a specific activity of 1,400,000 hemolytic units per mg of protein.

ELISA. The ELISA has been described in detail previously (5, 7). Briefly, cobalt-irradiated polystyrene microplates (Dynatech M 129 B; Plochingen, Federal Republic of Germany) were coated with antigen overnight at room temperature (22°C). The optimal coating dose was determined to be 5 μg/ml by antigen titration (Fig. 1). Patient sera were diluted 10^{-3} and incubated for 1 h at room temperature for immunoglobulin G (IgG) and IgA antibody determination and for 2 h at 37°C for IgM. Specific alkaline phosphatase-conjugated antisera (Orion Diagnostica, Finland) were added, and incubation was done overnight at room temperature. The ELISA titer was defined as the absorbance value at 405 nm multiplied by 10^3. A twofold increase between two samples was considered a significant rise. ELISA for measurement of antibodies against 13 different pneumococcal capsular polysaccharides has been described previously (10).

ELISA for IgG antibodies to *Staphylococcus aureus* alpha-toxin was essentially the same as for pneumolysin and has been described in detail previously (7).

Patients and sera. Two or more serum samples from 124 adult patients with pneumonia were examined (Table 1). Most of these patients have been described in detail previously (9, 12). Twenty-two patients had pneumococcal bacteremia. Pneumococcal pneumonia was diagnosed in another 47 patients by detection of pneumococcal capsular polysaccharide antigen in urine or serum (15 patients) by counterimmunoelectrophoresis (10) or in sputum (32 pa-
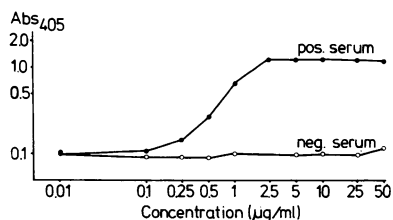
FIG. 1. Coating curve of pneumolysin antigen. Serum IgG was determined in one positive (pos.) and one negative (neg.) serum diluted 1,000 times. Abs, Absorbance.

tients) by coagglutination (11). Four of the 47 nonbacteremic pneumococcal pneumonia patients also showed at least a fourfold titer rise in complement-fixing antibodies (CF) against *Mycoplasma pneumoniae* antigen. Fifty-five patients had pneumonia caused by other agents. In patients with a primary diagnosis of *M. pneumoniae* infection ($n = 25$), the diagnosis was based on at least a fourfold titer rise in CF antibodies, but the diagnosis was also verified by sputum culture of *M. pneumoniae* in 22 of 25 patients. Diagnosis of *Chlamydia psittaci* infection ($n = 6$) was based on at least a fourfold rise in CF antibodies, as was the diagnosis of influenza A ($n = 6$). *Legionella pneumophila* infection ($n = 7$) was diagnosed by at least a fourfold titer rise by immunofluorescence. *Haemophilus influenzae* pneumonia ($n = 11$) was diagnosed by microscopy-directed semiquantitative sputum culture (12) and in most cases with support for the significance of the findings by at least a fourfold titer rise in CF antibodies against somatic *H. influenzae* antigen.

RESULTS

Healthy population of different ages. The occurrence of antibodies to pneumolysin was studied in 104 healthy individuals, 0.5 to 90 years of age. No marked age-correlated variations were noted in the adult population. Therefore, a common upper limit of normal values was used for these age groups. The limit used was the 95th percentile of the normal population, i.e., an ELISA titer of 500.

Patients with pneumococcal infection. Positive IgG antipneumolysin titers were found in 82% of patients with pneumococcal bacteremia, but in only 45% of patients with nonbacteremic pneumococcal pneumonia (Table 2). Most bacteremic patients (15 of 22) showed a significant rise in CF antibodies, although in 5 of 18 positive patients the titers did not reach the cutoff level for a positive (high) titer. For the

TABLE 1. Patient material studied by ELISA with purified pneumolysin as the antigen

Subject group (total no.)	No. of subjects	Median age in yr (range)
Pneumococcal pneumonia (69)		
Bacteremic	22	61 (35–84)
Nonbacteremic	47	67 (18–88)
Nonpneumococcal disease (55)		
M. pneumoniae	25	44 (19–90)
C. psittaci	6	43 (21–68)
Influenza A	6	66 (21–69)
L. pneumophila	7	58 (50–79)
H. influenzae	11	61 (46–80)
Healthy individuals	104	0.5–90

TABLE 2. Antibody response measured by ELISA to pneumolysin and capsular polysaccharides in 69 patients with pneumococcal pneumonia

ELISA antigen	No. (%) of patients with positive response	
	Bacteremic ($n = 22$)	Nonbacteremic ($n = 47$)
Pneumolysin		
IgG rise[a]	15 (68)	13 (28)
High titer[b]	13 (59)	21 (45)
Total	18 (82)	21 (45)
Capsular polysaccharides[c]		
IgG/IgM rise[a]	7/15 (47)	10/30 (33)

[a] At least a twofold rise.
[b] Above the upper limit of normal values, i.e., the 95th percentile for the healthy population.
[c] Types 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, and 23F, separately as well as in a pool.

nonbacteremic patients, on the other hand, antibody increases were infrequent (13 of 47), but all positive patients (21 of 21) reached high titer levels. In none of the groups could any further patient be diagnosed by use of IgM or IgA conjugates (data not shown). Less than half of the patients diagnosed by IgG antibody response to pneumolysin in the ELISA could be diagnosed by rises in IgG or IgM titer to capsular polysaccharides (Table 2).

The development of the IgG response in the 22 patients with bacteremic pneumococcal pneumonia is illustrated in Fig. 2. A significant titer rise was most often seen around day 10 in the course of the disease.

Seroconversion, i.e., a rise from negative to positive (>500) titer was also found at about day 10 in the whole group of 23 patients with pneumoccal pneumonia and rises in titer to positive levels.

Patients with other agents. Patients with pneumonia caused by other agents, i.e., *C. psittaci*, influenza A, *L. pneumophilia*, and *H. influenzae*, showed no titer rises or high titers to pneumolysin. In the patients with *M. pneumoniae*, however, six patients showed a significant rise in titer. Another two patients had high titers without titer rises, resulting in 8 of 25 (32%) cases being positive in this patient group. In all eight cases the diagnosis of mycoplasma infection was based on both serology by CF and on culture of *M.*
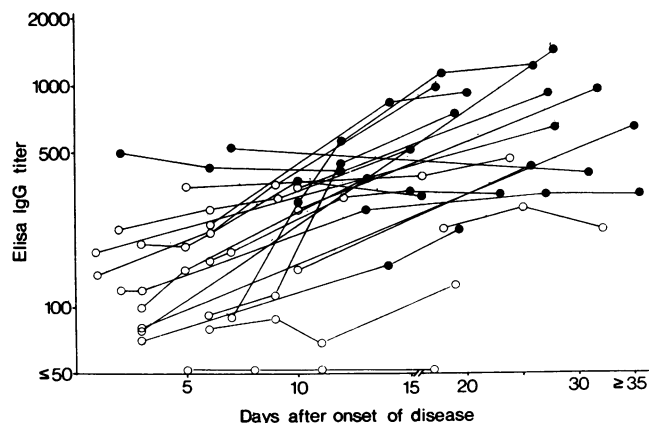

FIG. 2. IgG titers against pneumolysin in 22 patients with bacteremic pneumococcal pneumonia. Solid circles represent high or significantly rising titers.

pneumoniae from sputum samples. Paired sera from these patients were also assayed by ELISA for *S. aureus* α-toxin as the control antigen. All the patients, including the patients with *M. pneumoniae* infection, were negative in this test.

DISCUSSION

The occurrence of an antibody response against the type-specific capsular polysaccharide after a pneumococcal infection has long been known (3, 12). One evident problem in using this antigen for serological diagnosis is the existence of 83 different antigen types. Furthermore, despite the use of homotypic purified polysaccharides as antigen, an antibody response was detected in only half of the patients with pneumococcal pneumonia both by radioimmunoassay (16) and by ELISA (8, 10), at least partly because of the formation of antigen-antibody complexes (2). An antigen common to most or all strains and eliciting an antibody response would therefore be a better choice for diagnosis of pneumococcal disease. The pneumococcal hemolysin (pneumolysin) produced by most strains seemed to be a possible candidate for such an antigen (13). Previous investigators of antibodies against pneumolysin have used crude pneumolysin preparations and a neutralization test (4, 17, 19). The sensitivity and reproducibility of this assay were, however, not satisfactory (4, 17; G. Tunevall, personal communication). In the present study pneumolysin antibodies were measured by ELISA and with a highly purified antigen preparation.

Pneumolysin is good antigen candidate because of its occurrence in 99% of clinical isolates of *S. pneumoniae* (13). A possible problem might arise due to crossreaction with streptolysin 0, but in our hands 40 patients with high anti-streptolysin 0 titers were all negative in the pneumolysin ELISA. Further experiments on immunization of rabbits with purified preparations of streptolysin and pneumolysin indicate a relatively low degree (less then 5%) of cross-reactivity (K. Kanclerski, M. Granström, and R. Möllby, Acta Pathol. Microbiol. Scand., in press).

In patients with bacteremic pneumococcal pneumonia, a significant IgG increase could be noted in most cases. Increases in IgG titers mostly occurred at about day 10 in the course of the disease. In 5 of 18 patients a rise was found at a low antibody level. Four of these five patients had received antimicrobial chemotherapy within 3 days after onset of the disease. In nonbacteremic patients, a high convalescence-phase IgG level was found in half the cases, but significant increases in only one-fourth. Of the eight nonbacteremic patients with high but not significantly rising titers, five were admitted and first sampled more than 10 days after onset of the disease. The reason for the differences in antibody between bacteremic and nonbacteremic patients is probably the fact that bacteremic patients are more severely ill and therefore seek medical care sooner. Early administration of antimicrobial chemotherapy in bacteremic patients would thus interfere with the development of pneumolysin antibodies and concomitantly with the sensitivity of a serological assay. For nonbacteremic patients late-convalescence sera were most often not available. It is thus possible that a higher proportion than found here might have shown rising titers in the ELISA.

Finally, the sensitivity determination of a new diagnostic tool is dependent on the reliability of the other methods that the test is compared with. In this case it is natural that the results of the ELISA differ from those of the other tests, even with optimal sampling conditions. At least for cases

with increased titers in paired sera, the specificity of the ELISA should be superior to that of the other two methods.

Thirty patients with pneumonia caused by agents other than *S. pneumoniae* and *M. pneumoniae* were all negative in the pneumolysin ELISA test, which thus seems to be highly specific. However, 8 of 25 patients examined with serologically and culture-verified *M. pneumoniae* infection were positive in the pneumolysin ELISA. None of these eight patients responded promptly to penicillin, and six of them recovered only after erythromycin therapy. Six of the eight patients had rising titers, and two patients showed rises to very high titer levels. None of these patients showed antibody responses to another antigen investigated by ELISA, staphylococcal alpha-toxin. A polyclonal nonspecific stimulation during the mycoplasma infection therefore seems an unlikely explanation. Neither are there any known cross-reacting antigens between the two microorganisms that could explain this finding. Moreover, 4 of the 47 patients with nonbacteremic pneumococcal pneumonia had rising titers against *M. pneumoniae* antigen. Three of them had culture-positive sputum samples for *M. pneumoniae* as well. None of the four patients responded promptly to penicillin either, and three of them received erythromycin before cure was achieved. Kaijser et al. (8) also reported an antibody response against pneumococcal capsular polysaccharides in 6 of 23 (26%) patients with pneumonia caused by *M. pneumoniae*. Thus, our results seem to confirm earlier reports (3, 8) on the importance of dual infections with *M. pneumoniae* and *S. pneumoniae*.

In conclusion, ELISA for measurement of antibodies to highly purified pneumolysin seems to be a simple and specific method for the serological diagnosis of pneumococcal pneumonia. With this one antigen and with IgG antibody determination alone, a majority of patients with pneumococcal pneumonia could be diagnosed.

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